Original Study

Nucleolin Staining May Aid in the Identification of Circulating Prostate Cancer Cells

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Abstract

In advanced prostate cancer, there is a need for biomarkers to monitor response to therapy and determine prognosis. Current tests for circulating tumor cells (CTCs) rely on epithelial markers with limited sensitivity and specificity. We showed that the staining pattern of nucleolin, a protein associated with proliferative cells, aids in the classification of prostate cancer CTCs.

Introduction: Circulating tumor cells (CTCs) have great potential as circulating biomarkers for solid malignancies. Currently available assays for CTC detection rely on epithelial markers with somewhat limited sensitivity and specificity. We found that the staining pattern of nucleolin, a common nucleolar protein in proliferative cells, separates CTCs from white blood cells (WBCs) in men with metastatic prostate cancer. **Patients and Methods:** Whole peripheral blood from 3 men with metastatic prostate cancer was processed with the AccuCyte CTC system (RareCyte, Seattle, WA). Slides were immunostained with 4′,6-diamidino-2-phenylindole (DAPI), anti–pan-cytokeratin, anti-CD45/CD66b/CD11b/CD14/CD34, and anti-nucleolin antibodies and detected using the CyteFinder system. DAPI nucleolin colocalization and staining pattern wavelet entropy were measured with novel image analysis software. **Results:** A total of 33,718 DAPI-positive cells were analyzed with the novel imaging software, of which 45 (0.13%) were known CTCs based on the established AccuCyte system criteria. Nucleolin staining pattern for segmentable CTCs demonstrated greater wavelet entropy than that of WBCs (median wavelet entropy, 6.86×10^7 and 3.03×10^6, respectively; $P = 2.92 \times 10^{-22}$; approximated z statistic = 9.63). Additionally, the total nucleolin staining of CTCs was greater than that of WBCs (median total pixel intensity, 1.20×10^5 and 2.55×10^4 integrated pixel units, respectively; $P = 2.40 \times 10^{-21}$; approximated z statistic = 9.41). **Conclusion:** Prostate cancer CTCs displayed unique nucleolin expression and localization compared to WBCs. This finding has the potential to serve as the basis for a sensitive and specific CTC detection method.

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Introduction

Cancer cells that have entered the circulation of patients with solid cancers are called circulating tumor cells (CTCs). Because they are believed to represent a snapshot of the metastatic cascade, many studies have focused on CTC enumeration as a biomarker of metastatic disease.[1-4] Additionally, as a result of the repeatability and ease of blood sample collection, CTC analysis is viewed as reference standard of biomarkers to monitor response to therapy.[5-8] Despite the obvious clinical utility of CTC-based biomarkers, there are a number of technical challenges limiting the applicability of CTCs in clinical practice, most notably the rarity of CTCs in peripheral blood, where they circulate at a concentration on the order of ~1 CTC per 10^7 white blood cells (WBCs).[9] To overcome the low abundance of CTCs in blood, many technologies have been developed to enrich CTCs from whole blood.[10-16] These selection-based assays often use immunomagnetic enrichment of CTCs using epithelial cell surface markers, namely EpCAM.[15,17,18] The most prominent example of EpCAM-based CTC isolation is the CellSearch System (Janssen Diagnostics, Raritan, NJ), which is cleared by the US Food and Drug Administration for assessing prognosis in metastatic breast, prostate, and colorectal cancer patients.[18] Despite the successes of EpCAM-based CTC enrichment systems, data

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demonstrate that EpCAM expression is not limited to CTCs and that EpCAM may be expressed on cells of hematopoietic lineage.[19-22] Furthermore, as cancer cells develop a more aggressive phenotype (eg, after multiple lines of therapy), they tend to decrease expression of epithelial markers and may increase mesenchymal and stem-cell–like markers.[23-26]

To overcome the issues associated with the use of biomarkers for CTC isolation, selection-free methods for CTC detection utilizing high-throughput microscopy have been developed. Two prominent examples include the Epic Sciences system (Epic Sciences, San Diego, CA) and the AccuCyte and CyteFinder system (RareCyte, Seattle, WA).[27,28] Despite the advantages of these selection-free systems, these technologies still rely on biomarkers to detect and validate candidate CTCs. The reliance on specific biomarkers for CTC detection renders the systems only as sensitive and specific as the biomarkers used for detection. Standard markers used for high-throughput microscopy-based CTC detection systems include a nuclear marker, a counterstain marker (usually CD45; for denoting WBCs), and one or several epithelial markers including cytokeratins (hereafter pan-cytokeratin, or PanCK) and EpCAM.[29] To improve the diagnostic accuracy of these assays, there is a need for additional markers with which to positively identify candidate CTCs.

Nucleolin is a highly expressed nucleolar protein involved in ribosome maturation and gene expression with the ability to redistribute across cellular compartments.[30,31] Though the Gleason grading system for prostate cancer relies on cellular architecture, it is an outlier in that the majority of pathologic cancer grading systems depend on nuclear and nucleolar appearance.[32,33] For example, the International Society of Urological Pathology recommends a nucleolar grade for clear-cell and papillary renal-cell carcinoma.[33] Furthermore, it has been shown that nucleolin expression level and intracellular localization can be used to identify cancer cells in several settings such as non–small-cell lung cancer, ependyoma, and cervical squamous-cell carcinoma.[34-36] Accordingly, nucleolin has also been implicated in the processes of lymphangiogenesis and microtubule stabilization.[31,37]

In the present report, we investigated the utility of nucleolin as a biomarker of CTCs. We found that quantitation of this biomarker may improve confidence in CTC identification in men with metastatic prostate cancer.

Patients and Methods

Sample Collection

Peripheral blood from 3 men with metastatic prostate cancer was drawn into RareCyte blood collection tubes and processed within 72 hours of collection. Two of the 3 men had bone metastasis, none had brain metastasis, and they received a variety of therapies. (All men received androgen deprivation therapy, 2 patients received abiraterone, and 1 patient was also treated with docetaxel, cabazitaxel, platinum-based chemotherapy, doxorubicin, and nivolumab.) Blood specimens were collected under a protocol approved by the Johns Hopkins Medicine institutional review boards.

Blood Sample Processing

Blood samples were transferred to positively charged slides using the RareCyte AccuCyte and CyteSealer systems per the manufacturer's protocol. Slides were stored at −20°C until staining.

Sample Staining

On the day of staining, slides were fixed in 10% neutral buffered formalin (Sigma-Aldrich, St Louis, MO) for 60 minutes at 25°C. Excess formalin was neutralized with two 5-minute washes in Tris-buffered saline (Quality Biological, Gaithersburg, MD). Staining was performed using a proprietary custom reagent kit developed by RareCyte, which includes 4′,6-diamidino-2-phenylindole (DAPI; nuclear stain), anti-PanCK (epithelial marker), and anti-CD66b/CD45 (counterstain channel). This was modified to include a rabbit anti-nucleolin polyclonal antibody (Abcam, Cambridge, MA; rabbit polyclonal, 1:200) as well as the following additional phycoerythrin-labeled counterstain markers: anti-CD14-PE (Biolegend, San Diego, CA; clone M5E2, 1:200), anti-CD34-PE (Biolegend; clone 581, 1:200), and anti-CD11b-PE (Biolegend; clone M1/70, 1:200). Slides were otherwise stained according to manufacturers' protocols. Stained slides were mounted with Fluoromount aqueous mounting medium (Sigma-Aldrich) and allowed to dry overnight at 25°C.

Slide Scanning

Stained slides were scanned on the RareCyte CyteFinder high-throughput microscopy system with system ranking derived from "OR" logic of cytokeratin or nucleolin positivity. Candidate CTCs were identified from top-ranked cells by a single trained technician and reimaged with a high-magnification objective (400× total magnification) with the following exposure times: DAPI, 0.008 seconds; PanCK, 0.100 seconds; CD45/CD66b/CD11b/CD14/CD34 (counterstain channel), 0.100 seconds; and nucleolin, 0.100 seconds. Per the established AccuCyte protocol, prostate CTCs were identified as cells that had a DAPI-positive nucleus with a diameter of $\geq$ 4 μm, exhibited cytokeratin staining covering or surrounding $\geq$ 50% of the nucleus, and lacked staining in the counterstain channel (CD45/CD66b/CD11b/CD14/CD34), with all final calls made by a human researcher. Notably, nucleolin staining was not used as a criterion to identify CTCs. WBCs were identified as cells with a DAPI-positive nucleus with positive surface staining in the counterstain channel. Images were saved as 16-bit multichannel TIFF files.

Image Preprocessing

Multichannel TIFF files were split and saved with grayscale JPEG encoding (8-bit images) using Fiji software.[38]

CTC Image Analysis

Split-channel images were processed with a graphical program written with Matlab R2016a with the Image Processing Toolbox and Wavelet Toolbox (MathWorks, Natick, MA). DAPI grayscale images were loaded and segmented using a modified watershed algorithm. Region properties of segmented DAPI objects were used to define potential cells. Nucleolin staining within each DAPI mask was computed. Last, disorder of the nucleolin staining pattern within each DAPI mask was assessed by calculating the Shannon wavelet entropy of each 2-dimensional nuclear image.[39]

Data Analysis

Extracted image data was analyzed and plotted using the Statistics Toolbox in Matlab R2016a (MathWorks). *P* values were computed